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(54) Title: ASSAY FOR INCREASED INVASIVENESS OF EPITHELIAL CELLS

(57) Abstract

A method for determining relative invasiveness of an epithelial tumor, which comprises determining a prognostic amount of a prognostic marker selected from the group consisting of E-cadherin and α-catenin in a cell sample obtained from a cell source potentially containing cells of the epithelial tumor and comparing the prognostic amount to a normal amount of the prognostic marker in the cell source, wherein when the prognostic amount is less than the normal amount, the sample is indicative of increased invasiveness potential of the cells of the epithelial tumor. The method can also be used to monitor the effectiveness of treatment regimens and to screen candidate compounds for use in the treatment of epithelial tumors.

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ASSAY FOR INCREASED INVASIVENESS OF EPITHELIAL CELLS

INTRODUCTION

Technical Field

This invention is in the field of evaluation of tumor cells for indications of invasiveness and thus of prognosis of the disease, as a guide for physicians to use in selection or evaluation of treatment.

Background

Prostate cancer is one of the most common malignancies in men, and most patients will succumb to their disease once it has metastasized. For a discussion of prostate cancer, see Boring, C.C., Squires, T.S., and Tong, T. Cancer Statistics, 1993. CA-Cancer J Clin., *43:* 7-26, 1993. Improvements in the diagnosis of prostate cancer have now resulted in screening programs that reveal many more organ-confined lesions, with a concomitant increase in the number of radical surgeries. Whether this truly improves overall survival is not clear because of the unpredictable biological potential of these tumors. Not every prostate cancer will progress, as indicated in a number of studies, such as Johansson, J-E., Adami, H-O., Andersson, S-O., Bergström, R., Krusemo, U.B., and Kraaz, W. Natural history of localized prostatic cancer: a population-based study in 223 untreated patients. Lancet, *i:* 799-803, 1989 and Adolfsson, J., Rönström, L., Carstensen, J., Löwhagen, T., and Hedlund, P.O. The natural course of low grade, non-metastatic prostatic carcinoma. Br. J. Urol., *65:* 611-614, 1990. On the other hand, metastases still develop in a considerable group of patients with localized disease that are treated radically, see for example, Bostwick, D.G., Graham, S.D., Napalkov, P., Abrahamsson, P.-A., di Sant'Agnese, P.A, Algaba, F., Hoisaeter, P.A, Lee, F., Littrup, P., Mostofi, F.K., Denis, L, Schröeder, F., and Murphy, G.P. Staging of early prostate cancer: a proposed tumor volume-based prognostic

index. Urology, *41:* 403-411, 1993. The value of grade and clinical stage of the disease is controversial, as discussed in George, N.J.R. Natural history of localized prostatic cancer managed by conservative therapy alone, Lancet, *i:* 494-497, 1988 and Grayhack, J.T., and Assimos, D.G. Prognostic significance of tumor grade and stage in the patient with carcinoma of the prostate, Prostate, *4:* 13-31, 1983. This is true even though the pathological stage has been indicated in other studies to be one of the best prognostic factors available to date (see Morton, R.A., Steiner, M.S., and Walsh, P.C. Cancer control following anatomical radical prostatectomy: an interim report. J. Urol., *145:* 1197-1200, 1991). Clearly, methods that allow more accurate prediction of the clinical behavior are urgently needed.

In this respect the identification of molecular prognostic markers has now gained considerable attention, albeit that few of these have yet established clinical value. One of these markers, the epithelial cell adhesion molecule, E-cadherin, is of particular interest since it can function as invasion suppressor gene (see Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science (Washington DC), *251:* 1451-1455, 1991; Mareel, M.M., Behrens, J., Birchmeier, W., De Bruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C., and Van Roy, F.M. Down regulation of E-cadherin expression in Madin Darby canine kidney (MDKC) cells inside tumors of nude mice. Int. J. Cancer, *47:* 922-928, 1991; Vleminckx, K., Vakaet, L. Jr., Mareel, M., Fiers, W., and Van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role Cell, *66:* 107-119, 1991). In normal physiological conditions, E-cadherin plays an important role in embryonic development, morphogenesis and maintenance of epithelial integrity (see Takeichi, M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development, *102:* 639-655, 1988). Furthermore, loss of E-cadherin expression correlated well with the *in vitro* invasive phenotype of cancer cell lines (see Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., and Birchmeier, W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol., *113:* 173-185, 1991 and Bussemakers, M.J.G., Van Moorselaar, R.J.A., Giroldi, L.A., Ichikawa, T., Isaacs, J.T., Takeichi, M., Debruyne, F.M.J., and Schalken J.A. Decreased expression of E-cadherin in the progression of rat

prostatic cancer. Cancer Res., *52:* 2916-2922, 1992). Also, in human cancers E-cadherin expression correlated inversely with tumor grade. Recently we demonstrated a statistically significant correlation between aberrant expression of E-cadherin and increased grade of human prostate cancer. The staining pattern and intensity of all the well differentiated tumors (Gleason score 4 and 5) were uniformly positive, while all of the poorly differentiated and undifferentiated tumors (Gleason score 9 and 10) had aberrant E-cadherin expression comprising either heterogeneous or negative patterns (see Umbas, R., Schalken, J.A., Aalders, T.W., Carter, B.S., Karthaus, H.F.M., Schaafsma, H.E., Debruyne, F.M.J., and Isaacs, W.B. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res., *52:* 5104-5109, 1992). However, prior to the studies leading to the present invention it was not clear whether aberrant E--cadherin immunoreactivity had predictive value for the clinical course in patients with carcinoma of the prostate. In a study leading to the present invention we evaluated the prognostic value of E-cadherin expression in addition to tumor grade and stage in 89 patients ,with prostate cancer and have demonstrated clinical significance of the relationship.

The potential relation between E-cadherin expression and grade of tumor has been discussed for several carcinomas. See, for example, Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., Jahnke, K., and Birchmeier, W. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res., *51:* 6328-6337, 1991, Shimoyama, Y., and Hirohashi, S. Cadherin intercellular adhesion molecule in hepatocellular carcinomas: loss of E-cadherin expression in an undifferentiated carcinoma. Cancer Lett., *57:1* 31-135, 1991., Shimoyama, Y., and Hirohashi, S. Expression of E- and P-cadherin in gastric carcinoma. Cancer Res., *51:* 2185-2192, 1991, Van der Wurff, A.A.M., Ten Kate, J., Van der Linden, E.P.M., Dinjens, W.N.M., Arends, J-W., and Bosman, F.T. L-CAM expression in normal, premalignant, and malignant colon mucosa, J. Pathol., *168:* 287-291, 1992, Inoue, M., Ogawa, H., Miyata, M., Shiozaki, H., and Tanizawa, O. Expression of E-cadherin in normal, benign, and malignant tissues of female genital organs, Am. J. Clin. Pathol., *98:* 76-80, 1992 and Bringuier, P.P., Umbas, R., Schaafsma,

H.E., Karthaus, H.F.M., Debruyne, F.M.J., and Schalken, J.A. Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. Cancer Res., *53:* 3241-3245, 1993. An initial study relating to prostate cancer was published from the laboratories of the present inventors (Umbas, R., Schalken, J.A., Aalders, T.W., Carter, B.S., Karthaus, H.F.M., Schaafsma, H.E., Debruyne, F.M.J., and Isaacs, W.B. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res., *52:* 5104-5109, 1992).

Recently we found significant correlations between aberrant E-cadherin expression and survival in patients with prostate cancer. The prognostic value seemed to be independent of tumor stage since E-cadherin immunohistochemistry still had its prognostic value when we stratified the group of patients with localized disease and the group of patients with tumors that extended beyond the prostatic capsule. However, not every patient with normal E-cadherin expression showed better survival. Other studies also reported that some E-cadherin-expressing carcinomas often cause metastasis. These observations suggest that there might be some mechanism in these cancer cells capable of suppression of E-cadherin function even though the protein is present as demonstrated immunohistochemically (Shimoyama, Y., and Hirohashi, S. Expression of E- and P-cadherin in gastric carcinoma, Cancer Res., 51: 2185-2192, 1991; Oka, H., Shiozaki, H., Kobayashi, K., Tahara, H., Tamura, S., Miyata, M., Doki, Y., Iihara, K., Matsuyoshi, N., Hirano, S., Takeichi, M., and Mori T. Immunchlstochemical evaluation of E-cadherin adhesion molecule expression in human gastric cancer. Virchows Arch. A. Patho. Anat., 421: 149-156, 1992). Morton et al. (Morton, R.A., Ewing, C.M., Nagafuchi, A., Tsukita, S., and Isaacs, W.B. Reduction of E-cadherin levels and deletion of thte a-catenin gene in human prostate cancer cells, Cancer Res., 53: 3585-3590, 1993) reported that loss of normal E-cadherin function in prostate cancer cell lines can occur through mutational inactivation of the α-catenin gene. Recently, Kadowaki et al. (Kadowaki, T., Shiozaki, H. Inoue, M., Tamura, S., Oka, H., Doki, Y., Iihara, K. Matsui, S., Iwazawa, T., Nagafuchi, A., Tsukita, S., Mori, T. E-cadherin and a-catenin expression in human esophegeal cancer, Cancer Res., 54: 291-296, 1994) have independently reported that α-catenin

immunohistochemistry has additional prognostic value for oesophageal carcinoma. This specification describes our earlier investigations into the relationship between E-cadherin and α-catenin expression in prostate cancer patients.

SUMMARY OF THE INVENTION

Prostate cancers specimens from 89 patients were evaluated immunohistochemically for E-cadherin expression, and the results were related to histopathological grade, tumor stage, presence of metastases, and survival. A significant inverse correlation was found between E-cadherin expression and tumor grade. We also found significant correlations between E-cadherin expression and tumor stage and overall survival. Sixty-three percent of the tumors that extended beyond the prostate capsule (T3-4) *versus* 33% of the tumors confined to the prostate (T1-2) had aberrant expression (chi-square=8.1, $p<0.005$). Seventy-six percent of the primary tumors from patients that presented with metastases showed aberrant staining compared to 32% from patients without metastases (chi-square=14.9, $p<0.001$). The life table analysis showed a significantly higher survival rate for patients with normal staining compared to patients with aberrant expression (log rank test: chi-square=20.4, $p<0.001$). Moreover, abnormal expression of E-cadherin correlated significantly with progression after radical prostatectomy ($p < 0.005$). These results indicate that E-cadherin expression can serve as a prognostic indicator for the biological potential of prostate cancer and thus can function as an important prognostic tool.

Additional work in the laboratories of the inventors has demonstrated an even better correlation between presence of expression of α-catenin in prostate tumor cells and survival. Several patients in a clinical study showing poor clinical prospects but normal E-cadherin expression have been found to show aberrant α-catenin expression by immunohistochemistry. To determine the correlation between E-cadherin and α-catenin expression in prostate cancer, 20 patients treated by radical prostatectomy and 32 advanced stage patients were evaluated immunohistochemically for E-cadherin and α-catenin expression. The results were related to tumor grade and progression of the disease. Four patients in the radical prostatectomy group show aberrant E-cadherin and α-catenin expression, all of these

patients showed progression. The other 16 patients were free of progression and have both normal E-cadherin as well as α-catenin expression. In the advanced stage group, 4 of 13 patients with normal E-cadherin staining show aberrant α-catenin expression and 2 (50%) were progressed compared with only 22% progression in patients having both normal E-cadherin and α-catenin expression. The other 19 patients with aberrant E-cadherin and α-catenin staining have the poorest prognosis. These results indicate that loss of α-catenin immunoreactivity is one of the mechanisms responsible for the loss of mediated E-cadherin cell-cell adhesion function in human prostate cancer, and this will provide additional prognostic information for epithelial cancers in general.

Accordingly, the present invention provides a method for determining invasiveness of an epithelial tumor, which comprises determining a prognostic amount of a prognostic marker selected from the group consisting of E- cadherin and α-catenin in a cell sample obtained from a cell source potentially containing cells of said epithelial tumor and comparing said prognostic amount to a normal amount of said prognostic marker in said cell source, wherein when said prognostic amount is less than said normal amount, said sample is indicative of enhanced invasiveness potential of said ephithelial tumor. The method in particularly advantageous when α-catenin alone or in combination with E-cadherin is being monitored. These same markers can be followed to monitor treatment efficacy or to screen for new drugs that would be useful for the dedifferentiation of epithelial tumors.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood by reference to the following drawings, wherein:

Fig. 1 is a photomicrograph showing invasiveness of cells and corresponding labelling of such cells with an antibody to E-cadherin. The different panels of the figure are as follows: a, normal staining, in the well differentiated tumor staining is confined to the cell contacts. b, heterogeneous staining. Positive membranous staining on some of the cells whereas others are negative. c, completely negative staining, none of the tumor cells are stained.

Fig. 2 is a graph showing Kaplan-Meier overall survival rate related to E-cadherin expression. Bars, censured data. Log rank test: chi-square= 20.4, $p<0.001$.

Fig. 3 is a graph showing Kaplan-Meier progression free rate in patients after radical prostatectomy. Bars, censured data. Log rank test: chi-square= 9.4, $P<0.005$.

Fig. 4 is a graph showing Kaplan-Meier survival curves. Bars, censured data. 3, 3-year survival related to E-cadherin expression in patients with primary TUR-P (TUR-P, transurethral resection of the prostate): Log rank test: chi-square= 15.1, $p<0.001$. b, 3-year survival of patients with TUR-P after recurrent disease according to E-cadherin expression: Log rank test: chi-square= 1.2, $p<0.3$.

DESCRIPTION OF SPECIFIC EMBODIMENTS

To evaluate the potential use of E-cadherin immunohistochemistry as a prognostic factor for prostatic cancer patients, and thus the epthelial cancers in general, we analyzed 89 prostate cancer specimens. We found that aberrant E-cadherin expression (heterogeneous, cytoplasmic or negative) was present in 33% (15 of 46) of the tumors that were clinically found to be organ confined (T1-2), and in 63% (27 of 43) of the lesions that extended beyond the prostatic capsule (T3-4). Moreover, 76% of the patients that were diagnosed with metastatic disease showed aberrant E-cadherin expression patterns. This is in good agreement with non-clinical studies by Schipper et al. (Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., Jahnke, K., and Birchmeier, W. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res., *51:* 6328-6337, 1991) and Mattijssen et al. (Mattijssen, V., Peters, H., Schalkwijk, L., Manni, J.J., de Mulder, P.H.M., and Ruiter, D.J. E-cadherin expression in head and neck squamous cell carcinoma. Int. J. Cancer, 55:580-585, 1993) on squamous cell carcinoma of the head and neck that revealed a close correlation between aberrant E-cadherin expression and grade, and with presence of lymph node metastases. Furthermore, we now have discovered that overall survival correlated significantly with aberrant E-cadherin expression.

When we stratified according to treatment, *i.e.*, radical prostatectomy for localized disease *versus* palliation in case of locally or distantly metastatic prostate cancer, E--cadherin immunohistochemistry still had prognostic value. Progression after radical prostatectomy occurred in 67% (10 of 15) patients with aberrant E-cadherin expression, and only in 4% (1 of 27) of patients with normal E-cadherin staining. The correlation between aberrant E-cadherin expression and poor prognosis was also evident within the group of patients with tumors that extended beyond the prostatic capsule. Thus, this is the first study that demonstrates E-cadherin immunohistochemistry to be of value as prognostic factor in following the course of prostate cancer, and thus is a valuable indicator for epithelial cancers in general.

It should be noted that E-cadherin expression was not indicative for progression in all of the patients. This may be explained by impaired catenin function through which E-cadherin is anchored to the cytoskeleton. The anchoring function has been studied, but the prognostic relationships have not previously been demonstrated. See, for example, Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science (Washington DC), *251:* 1451-1455, 1991, Giroldi, L.A., and Schalken, J.A. Decreased expression of the intercellular adhesion molecule E-cadherin in prostate cancer: biological significance and clinical implications. Cancer and Metastasis Reviews, *12:* 29-37, 1993 and Shimoyama, Y., Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S., and Hirohashi, S. Cadherin dysfunction in a human cell line: possible involvement of loss of α-Catenin expression in reduced cell-cell adhesiveness. Cancer Res., *52:* 5770-5774, 1992.

Other mechanisms have also been proposed that could explain this phenomenon. Recently Morton et al. found that in a prostate cancer cell line (PC3) having impaired E-cadherin function could be explained by homozygous deletion of the alpha-catenin gene (Morton, R.A., Ewing, C.M., Nagafuchi, A., Tsukita, S., and Isaacs, W.B. Reduction of E-cadherin levels and deletion of the α-catenin gene in human prostate cancer cells. Cancer Res., *53:* 3585-3590, 1993). This phenomenon might explain the discrepancy between normal E-cadherin expression and the presence of metastasis at time of diagnosis. Another mechanism that can lead to dysfunction of cadherin mediated interaction are increased tyrosine

phosphorylation of β-catenin (see Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M., and Birchmeicr, W. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. J. Cell Biol., *120:* 757766, 1993). Alternatively, there may be mutational inactivation of the E-cadherin function itself. The latter possibility is particularly interesting since the chromosomal segment to which E-cadherin is mapped (16q21) is frequently lost in prostate cancer development (see Carter, B.S., Ewing, C.M., Ward, W.S., Treiger, B.F., Aalders. T.W., Schalken, J.A., Epstein, J.I., and Isaacs, W.B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc. Natl. Acad. Sci. USA., *87:* 8751-8755, 1990 and Bergerheim, U.S.R. Kunimi, K., Collins, V.P., and Ekman, P. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. Genes, Chromosomes & Cancer, *3:* 215-220, 1991). As yet the mutational inactivation of E-cadherin function has not been found in human prostate cancer, and only reported for a subset of endometrial carcinomas and gastric cancers (see Risinger, J.I., Kohler, M.F., Berchuck, A., and Boyd, J. Mutation of the E-cadherin gene in endometrial carcinoma (abstract). Proceedings annual meeting of the AACR, *34:* 540, 1993 and Becker, K.F., Atkinson, M.J., Reich, U., Huang, H.H., Nekarda, H., Siewert, J.R., Höfler, H. Exon skipping in the E-cadherin gene transcript in metastatic human gastric carcinomas. Hum. Mol. Genetics, *2:* 803-804, 1993).

To investigate these possibilities, we also carried out studies of α-catenin. We found significant correlation between α-catenin and E-cadherin expression (Example 2: Table 4; x^2 - 38.2). There were 3 staining combinations: first, normal expression of both E-cadherin and α-catenin; second, normal E-cadherin expression and aberrant α-catenin staining; and third, both aberrant expression of E-cadherin and α-catenin. Patients who show normal immunoreactivity for both of these molecules have significantly better prognosis than patients having aberrant α-catenin expression, even if the E-cadherin expression was normal (Table 5; x^2 - 28.2). In a radically treated group, 4 patients showed aberrant E-cadherin and α-catenin expression, and all of these patients progressed (i.e., their cancer progressed adversely) within 8 to 37 months. The other 16 patients of this group showed

normal expression of both E-cadherin and α-catenin, and no progression could be detected in a follow up of 40 months (mean; range, 20-65 months).

Thirteen patients with advanced disease had normal E-cadherin expression; nine of these patients had also normal α-catenin expression and 2 (22%) of them died of the disease, while 2 (50%) of the 4 patients in this group which had aberrant α-catenin expression died. Of the other 19 patients who had both aberrant expression of E-cadherin and α-catenin, only 3 (16%) were alive without progression. The other 16 (84%) either developed progression (1 patient) or died of the disease. Thus, the combination of following both markers provided a better means of following patient prognosis than was possible for either marker alone, while α-catenin alone provided better prognosis than E-caderin alone, at least for some patients.

Thus, irrespective of the exact mechanism, the loss of cell adhesion and the resulting increase in tumor invasiveness resulting from impaired E-cadherin function is a marker of the loss of epithelial integrity, so that monitoring decrease of expression of either E-cadherin or α-catenin (or preferably both) in epithelial cells provides a valuable technique for following the progression of epithelial cancer.

Definition of Terms

In order to avoid awkward language, the molecule being detected is referred to as a "prognostic marker," whether that molecule is E-catenin or α-cadherin.

E-cadherin is an epithelial cell-cell adhesion molecule that is located on the surface of epithelial cells. It is a well known and defined molecule and has been fully sequenced. Numerous publications describing E-cadherin are provided in the background section of this specification.

The E-cadherin gene in humans is located on the long arm of chromosome 16 at position 22.1 and is described in a number of publications, including Mansouri et al. Differentiation 38:67-71, 1988. The sequence of the E-cadherin gene and of the cDNA for the expressed polypeptide have been published and are present in several databases, including the EMBL, GENBANK, and DDBJ databases.

α-Catenin is a cytoplasmic epithelial cell polypeptide that acts cooperatively to anchor E-cadherin to the surface of epithelial cells by interactions with the

cytoplasmic domain of E-cadherin. It is a well known and defined molecule and has been fully sequenced. Numerous publications describing α-catenin are provided in the background section of this specification.

The α-catenin gene in humans is located on the long arm of chromosome 5 at position 21-31 and is described in a number of publications (see for example, Oda et al., Biochem. Biophys. Res. Comm. 193:897-904 (1993) and Claverie et al. Genomics 15:13-20 (1993)). The sequence of the α-catenin gene and of the cDNA for the expressed polypeptide have been published and are present in several databases, including the EMBL, GENBANK, and DDBJ databases.

Two sequences of amino acids (polypeptides) are "homologous" if a first sequence at least 10 amino-acid residues in length can be substantially matched on an amino-acid to amino-acid basis with a second sequence, with no more than 20% (two in the case of the minimal length of 10) missing ("gaps") or additional ("inserts") amino acids being allowed in the matching region of the second sequence. More preferably, the gaps and inserts are less than 10%, even more preferably less than 5%, of the total sequence. Homologies are generally stated in percentages; for example, "at least 80% homology" to a twenty amino acid first sequence would require that the second sequence contain at least 16 identically ordered amino acids, with no more than four gaps or four inserts in the second sequence.

Two nucleic acid fragments are "homologous" if they are capable of hybridizing to one another under hybridization conditions described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor NY 1982, pp. 320-323. However, by using the following wash conditions -- 2 x SCC, 0.1% SDS, room temperature twice, 30 minutes each; then 2 x SCC, 0.1% SDS, 50°C once, 30 minutes; then 2 x SCC, room temperature - twice, 10 minutes each -- homologous sequences can be identified that contain at most about 25-30% basepair mismatches. More preferably, "homologous" nucleic acid sequences contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches. These degrees of homology can be selected by using more stringent wash conditions for identification of clones from gene libraries (or other sources of genetic material), as is well known in the art. The definition of

homology above applies generally when percent homology is not stated for homologous sequences. Alternatively, homologous nucleic acid strands contain the idential numerical values (for base pairs rather than amino acid residues) that are stated above for amino acid sequences when percent homologies are stated.

A DNA or RNA fragment is "derived from" a prognostic-marker-encoding DNA or RNA sequence if it has the same or substantially the same basepair sequence as a region of the coding sequence for the entire prognostic marker molecule. A probe is typically complementary to such a fragment derived from a marker gene.

"Substantially the same" means, when referring to biological activities, that the activities are of the same type although they may differ in degree. When referring to amino acid or nucleotide sequences, substantially the same means that the molecules in question have similar biological properties and preferably have at least 90% homology in amino acid or nucleotide sequences. More preferably, the amino acid sequences are at least 95% identical. In other uses, "substantially the same" has its ordinary English language meaning.

A protein or peptide is "derived from" an prognostic marker molecule if it has the same or substantially the same amino acid sequence as a region of the prognostic marker molecule. Fragments derived from the natural markers are one type of derivative. Such proteins and peptides are useful in the preparation of antibodies specific for the corresponding diagnostic marker. Production of antibodies specific for prognostic markers is discussed elsewhere in this specification. Minor amino acid variations from the natural amino acid sequence of prognostic markers are contemplated as being encompassed by the term prognostic marker; in particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) non-polar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding properties of the resulting molecule, especially if the replacement does not involve an amino acid at a site involved in induction of an antibody. Whether an amino acid change results in a functional peptide useful for production of an antibody that reacts with an actual prognostic marker can readily be determined by assaying the specific binding properties of the prognostic marker polypeptide derivative or antibody produced.

Sources and preparation of compositions of prognostic markers of the invention

Prognostic markers of the invention for use in the preparation of antibodies and as diagnostic standards can readily be purified from natural sources and from cells genetically modified to produce prognostic marker or polypeptide derivatives or fragments thereof. While prognostic markers of the invention preferably means human prognostic markers, prognostic markers of mammals, e.g. murine, porcine, equine or bovine, are included within the definition of prognostic markers (as long as they comply with the required degree of homology), and tissues from such animals can serve as sources of prognostic marker. Homologies are high, in excess of 95% for various regions of probes and markers derived from markers obtained from different vertebrate species.

Crude polypeptide preparations can be purified by numerous techniques, such as by affinity chromatography using a monoclonal antibody specific for the prognostic marker being isolated. Such antibodies are commercially available (for example anti E-cadherin from Euro-diagnostica BV, Apeldoorn, the Netherlands or HECD-1 from Takara, Berkeley, USA). In addition to the use of affinity chromatography, prognostic markers and polypeptide derivatives thereof can be purified by a variety of other widely known protein purification techniques (either alone or in combination) including immunoprecipitation, gel filtration, ion exchange chromatography, chromatofocusing, isoelectric focusing, selective precipitation, electrophoresis, and the like (for example, see Nagafuchi et al., Cell 65:849-857,

1991; Napolitano et al., J. Cell Biol. 113:893-905, 1991; Takeichi, Development, 102:939-655, 1988 and references therein).

Fractions isolated during purification procedures can be analyzed for the presence of prognostic marker or polypeptide derivatives of prognostic marker by immunoassays employing prognostic-marker-specific antibodies or prognostic-marker-specific bioassays.

Production of Prognostic Marker by Genetic Engineering

Since the gene sequence of both E-cadherin and α-catenin have been published and are readily availiable through commerical databaaes, such as GENBANK, the genetic information necessary for production of markers by genetic engineering (or for production of probes for analysis) is readily available. This genetic information can be used directly (e.g., for production of probes by automated chemical synthesis) or indirectly (e.g., for isolation of marker genes or fragments thereof from natural sources).

Isolation of nucleotide sequences encoding a prognostic marker generally involves creation of either a genomic library prepared from cells encoding prognostic marker or preparation of a cDNA library from RNA isolated from cells expressing prognostic marker. It will generally be preferable to create a cDNA library for isolation of prognostic marker coding nucleotide sequences so as to avoid any possible problems arising from attempts to determine intron/exon borders. Genetic libraries can be made in either eukaryotic or prokaryotic host cells. Widely available cloning vectors such as plasmids, cosmids, phage, YACs and the like can be used to generate genetic libraries suitable for the isolation of nucleotide sequences encoding prognostic marker or portions thereof.

Useful methods for screening genetic libraries for the presence of prognostic marker nucleotide sequences include the preparation of oligonucleotide probes based on the N-terminus amino acid sequence information from purified prognostic marker or purified internal fragments of purified prognostic marker. By employing the standard triplet genetic code, oligonucleotide sequences of about 17 base pairs or longer can be prepared by conventional in vitro synthesis techniques so as to correspond to portions of prognostic marker for which the amino acid sequence has

been determined by N-terminus analysis. The resultant nucleic acid sequences can be subsequently labeled with radionuclides, enzymes, biotin, fluorescers, or the like, and used as probes for screening genetic libraries.

Additional methods of interest for isolating prognostic marker-encoding nucleic acid sequences include screening genetic libraries for the expression of prognostic marker or fragments thereof by means of prognostic marker-specific antibodies, either polyclonal or monoclonal. A particularly preferred technique involves the use of degenerate primers based on partial amino acid sequences of purified prognostic marker or on sequences from known related molecules and the polymerase chain reaction (PCR) to amplify gene segments between the primers. The gene can then be isolated using a specific hybridization probe based on the amplified gene segment, which is then analyzed for appropriate expression of protein. Nucleotide sequences encoding prognostic marker can be obtained from recombinant DNA molecules recovered from prognostic marker genetic library isolates. The nucleotide sequence encoding prognostic marker can be obtained by sequencing the non-vector nucleotide sequences of these recombinant molecules. Nucleotide sequence information can be obtained by employing widely used DNA sequencing protocols, such as Maxim and Gilbert sequencing, dideoxy nucleotide sequencing, and the like. Examples of suitable nucleotide sequencing protocols can be found in Berger and Kimmel, Methods in Enzymology Vol. 52, Guide to Molecular Cloning Techniques, (1987) Academic Press. Nucleotide sequence information from several recombinant DNA isolates, including isolates from both cDNA and genomic libraries, may be combined so as to provide the entire amino acid coding sequence of prognostic marker as well as the nucleotide sequences of introns within the prognostic marker gene, upstream nucleotide sequences, and downstream nucleotide sequences.

Nucleotide sequences obtained from sequencing prognostic marker specific genetic library isolates are subjected to analysis in order to identify regions of interest in the prognostic marker gene. These regions of interest include open reading frames, introns, promoter sequences, termination sequences, and the like. Analysis of nucleotide sequence information is preferably performed by computer. Software suitable for analyzing nucleotide sequences for regions of interest is

commercially available and includes, for example, DNASIS ~ (LKB). It is also of interest to use amino acid sequence information obtained from the N-terminus sequencing of purified prognostic marker when analyzing prognostic marker nucleotide sequence information so as to improve the accuracy of the nucleotide sequence analysis.

Isolated nucleotide sequences encoding prognostic marker can be used to produce purified prognostic marker or fragments thereof by either recombinant DNA methodology or by in vitro polypeptide synthesis techniques. By "purified" and "isolated" is meant, when referring to a polypeptide or nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term "purified" as used herein preferably means at least 95% by weight, more preferably at least 99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000, can be present).

A significant advantage of producing prognostic marker by recombinant DNA techniques rather than by isolating prognostic marker from natural sources is that equivalent quantities of prognostic marker can be produced by using less starting material than would be required for isolating the binding protein from a natural source. Producing prognostic marker by recombinant techniques also permits prognostic marker to be isolated in the absence of some molecules normally present in cells that naturally produce prognostic marker. Indeed, prognostic marker compositions entirely free of any trace of human protein contaminants can readily be produced since the only human protein produced by the recombinant non-human host is the recombinant prognostic marker. Potential viral agents from natural sources are also avoided. It is also apparent that recombinant DNA techniques can be used to produce prognostic marker polypeptide derivatives that are not found in nature, such as the variations described above.

Prognostic marker and polypeptide derivatives of prognostic marker can be expressed by recombinant techniques when a DNA sequence encoding the relevant molecule is functionally inserted into a vector. By "functionally inserted" is meant in proper reading frame and orientation, as is well understood by those skilled in

the art. When producing a genetic construction containing a complete prognostic marker reading frame, the preferred starting material is a cDNA library isolate encoding prognostic marker rather than a genomic library isolate. Typically, the prognostic marker gene will be inserted downstream from a promoter and will be followed by a stop codon, although production as a hybrid protein followed by cleavage may be used, if desired. In general, host-cell-specific sequences improving the production yield of prognostic marker and prognostic marker polypeptide derivatives will be used and appropriate control sequences will be added to the expression vector, such as enhancer sequences, polyadenylation sequences, and ribosome binding sites.

Once the appropriate coding sequence is isolated, it can be expressed in a variety of different expression systems. Only one such system will be described here in detail, as the present invention is not directed to the production or use of E-cadherin or α-catenin, merely to the detection of these previously known materials and their use in predicting prognosis of epithelial tumor progression.

Mammalian Expression Systems for Production of Markers

Since E-cadherin and α-catenin are mammalian proteins, expression in a mammalian expression system is preferred. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, typically located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation (Sambrook et al. (1989) "Expression of Cloned Genes in Mammalian Cells." In Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratories).

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallotheionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will typically increase expression levels. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter [Maniatis et al. (1987) Science 236:1237; Alberts et al. (1989) Molecular Biology of the Cell, 2nd ed.]. Enhancer elements derived from viruses may be particularly useful, because they typically have a broader host range. Examples include the SV40 early gene enhancer [Dijkema et al (1985) EMBO J. 4:761] and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus [Gorman et al. (1982b) Proc. Natl. Acad. Sci. 79:6777] and from human cytomegalovirus [Boshart et al. (1985) Cell 41:521]. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion [Sassone-Corsi and Borelli (1986) Trends Genet. 2:215; Maniatis et al. (1987) Science 236:1237].

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either in vivo or in vitro. The leader sequence fragment typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation [Birnstiel et al. (1985) Cell 41:349; Proudfoot and Whitelaw (1988) "Termination and 3' end processing of eukaryotic RNA. In Transcription and splicing (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) Trends Biochem. Sci. 14:105]. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 [Sambrook et al (1989) "Expression of cloned genes in cultured mammalian cells." In Molecular Cloning: A Laboratory Manual].

Some genes may be expressed more efficiently when introns (also called intervening sequences) are present. Several cDNAs, however, have been efficiently expressed from vectors that lack splicing signals (also called splice donor and acceptor sites) [see e.g., Gothing and Sambrook (1981) Nature 293:620]. Introns are intervening noncoding sequences within a coding sequence that contain splice donor and acceptor sites. They are removed by a process called "splicing," following polyadenylation of the primary transcript [Nevins (1983) Annu. Rev. Biochem. 52:441; Green (1986) Annu. Rev. Genet. 20:671; Padgett et al. (1986) Annu. Rev. Biochem. 55:1119; Krainer and Maniatis (1988) "RNA splicing." In Transcription and splicing (ed. B.D. Hames and D.M. Glover)].

Typically, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 [Gluzman (1981) Cell 23:175] or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a procaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 [Kaufman et al. (1989) Mol. Cell. Biol. 9:946 and pHEBO [Shimizu et al. (1986) Mol. Cell. Biol. 6:1074].

As alternatives to expression in mammalian systems, E-cadherin and/or α-catenin can also be produced in other expression systems, such as Baculovirus expression systems, bacterial expression systems, and yeast expression systems. The modifications of the above-described mammalian system for use with these other expression systems are well within the skill of those skilled in the art.

Diagnostic Applications of Prognostic Marker

Assays for prognostic marker molecules of the invention, as well as for the genetic material (especially mRNA) encoding the markers, can be used to follow the course of epithelial tumor progression, as well as to follow the course of treatment of such tumors.

Typically, methods for detecting cellular analytes such as prognostic marker proteins of the invention are based on immunoassays. Such techniques are well known and need not be described here in detail. In many cases the assays will be carried out on tissue samples rather than in solution, since the presence of the

prognostic markers is associated with cells that adhere to each other. However, it is also possible to assay clusters of cells not found as differentiated tissues (such as ascites tumors) as well as to assays for the presence of the markers (or their expression) by detecting, for example, the markers or mRNA encoding them in solution after the cells of a tissue (or other source) have been dispersed or even lysed. Thus, there is no limit to the cell source being assayed, although it is advantageous to make comparison of prognostic and normal amounts of marker expression using cells from the same cell source.

When tissues or cell clusters are being assayed, immunohistochemistry is a particularly useful type of assay, as it is possible to directly visualize the presence of the prognostic markers in the cells. A complete description of an immunohistochemical assay is given below (as an example of a qualitative assay). However, it will be recognized that any such example is merely one of a number of variations of immunohistochemistry. The invention is not limited to any specific assay.

Assays based on processes other than immunohistochemistry are also not limited to particular types of assays. Examples include both heterogeneous and homogeneous immunoassay techniques. Both techniques are based on the formation of an immunological complex between the binding protein and a corresponding specific antibody. Heterogeneous assays for prognostic marker typically use a specific monoclonal or polyclonal antibody bound to a solid surface. Sandwich assays are increasingly popular. Homogeneous assays, which are carried out in solution without the presence of a solid phase, can also be used, for example by determining the difference in enzyme activity brought on by binding of free antibody to an enzyme-antigen conjugate. A number of suitable assays are disclosed in U.S. Patent Nos. 3,817,837, 4,006,360, 3,996,345.

The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activate carboxyl, hydroxyl, or aldehyde group.

In a second diagnostic configuration, known as a homogeneous assay, antibody binding to an analyte produces some change in the reaction medium which can be directly detected in the medium. Known general types of homogeneous assays proposed heretofore include (a) spin-labeled reporters, where antibody binding to the antigen is detected by a change in reported mobility (broadening of the spin splitting peaks), (b) fluorescent reporters, where binding is detected by a change in fluorescence efficiency, (c) enzyme reporters, where antibody binding effects enzyme/substrate interactions, and (d) liposome-bound reporters, where binding leads to liposome lysis and release of encapsulated reporter. The adaptation of these methods to the protein antigen of the present invention follows conventional methods for preparing homogeneous assay reagents.

The assay can be either qualitative or quantitative. Since all cells normally contain both of the indicated prognostic markers and since even a single invasive tumor cell can cause deleterious clinical effects, any decrease in the amount or concentration of either of the prognostic markers is clinically significant.

A typical qualitative assay can be carried out using immunohistostaining. Indirect immunoperoxidase staining can be carried out as described in detail in Umbas, R., Schalken, J.A., Aalders, T.W., Carter, B.S., Karthaus, H.F.M., Schaafsma, H.E., Debruyne, F.M.J., and Isaacs, W.B. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer, Cancer Res., *52:* 5104-5109, 1992, using commercially available monoclonal antibodies, such as anti E-cadherin (Eurodiagnostica BV, Apeldoorn, The Netherlands) or HECD-1 (Takara, Berkeley, U.S.A.) monoclonal antibodies. Typically, surgical specimens to be assayed are snap frozen, sectioned to about 4-6 μm thickness, air-dried and stored at -20°C until used. One section from each sample is stained with hematoxylin and eosin to assess the histopathological grade according to Gleason (Gleason, in Urologic Pathology: The Prostate, M. Tannenbaum, ed., Philadelphia, Lea and Febiger, 1977, pp. 171-197). E-cadherin staining is localized on the membrane, particularly at areas of cell-cell contact, in normal cells. A typical technique for assessing the staining and thus the presence of the prognostic marker is to designate the stained cells as falling into one of three general categories: uniformly positive, uniformly negative, or heterogeneous (mixed

populations of positive and negative stained cells). Such a classification has previously been described for other cell types, as described by Schipper et al. (Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., Jahnke, K., and Birchmeier, W. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res., *51:* 6328-6337, 1991). Besides positive or negative staining, some tumors can show a cytoplasmic staining, which is also considered to be abnormal; such cells are included in the criteria for heterogeneous staining. Uniformly positive staining patterns are regarded as normal while uniformly negative and heterogeneous stainings are both considered as aberrant expression. Thus, the appearance of anything less than staining of all cells is an indication of increased relativeness invasiveness, with lesser (or no) staining indication relatively greater invasiveness.

When used as a quantitative analysis, a predetermined range of concentrations for the same cell source in the normal population is typically obtained by using the same assay technique that will be used in the application of the method to an individual being tested, in order to ensure the highest correlation. In preferred embodiments sufficient measurements are made in a normal population to produce a statistically significant range of normal values for the value to which a comparison will be made. Generally, the minimum concentration (or other amount) indicative of increased invasiveness is considered to be (for example) 1, 2, 3, or 4 standard deviations below the mean prognostic marker concentration for the normal population, for any given cell source.

It will be recognized by those familiar with statistics that the number of standard deviations used as an indication of increased invasiveness will be selected with an appropriate diagnosis goal in mind. For example, one standard deviation would encompass about 68% of normal samples; that is, 32% of normal samples would be expected to fall outside the lower and upper limits set by one standard deviation from the mean (16% would thus be expected to be below the selection limit). Thus, one standard deviation above the normal mean would not be used for definitive analysis for increased invasiveness, as it would include too many false positives. However, one standard deviation is appropriate for an assay that is

desired to sweep in for further evaluation all possible candidates who might be predisposed to metastasis, as in a patient with a personal or family history of metastasis. Two standard deviations from the mean would encompass about 95% of normal samples; three standard deviations, about 99%; four standard deviations, more than 99%. These levels are more appropriate generally, especially for samples from a cell source or patient that show a high coefficient of variance.

It is not necessary to express the lower limit of the indication of invasiveness (lower limit of the normal range) in standard deviations. Any other system that can be used to provide a statistically significant indication of invasiveness can be used. For example, the limit can be set to be a concentration (or other measure) that is at least as high as the 95th percentile concentration for normal patients for the same cell source.

<u>Specific Prognostic-marker-binding Molecules and Production of Anti-prognostic-marker Antibodies</u>

Prognostic-marker-specific binding molecules include polypeptides such as antibodies that are specific for the prognostic marker polypeptide containing the naturally occurring prognostic marker amino acid sequence. By "specific binding polypeptide" is intended polypeptides that bind with a prognostic marker and which have a measurably higher binding affinity for the target polypeptide, i.e., prognostic marker and polypeptide derivatives of prognostic marker, than for other polypeptides tested for binding. Higher affinity by a factor of 10 is preferred, more preferably a factor of 100. Binding affinity for antibodies refers to a single binding event (i.e., monovalent binding of an antibody molecule). Specific binding by antibodies also means that binding takes place at the normal binding site of the antibody (i.e., at the end of the arms in the variable region).

Prognostic markers, both glycosylated and unglycosylated (E-cadherin is normally glycosylated; α-catenin is not), or polypeptide derivatives thereof, may be used for producing antibodies, either monoclonal or polyclonal, specific to prognostic marker. By polypeptide derivatives is meant polypeptides differing in length from natural prognostic marker and containing five or more amino acids from the prognostic marker in the same primary order as found in the prognostic

marker as obtained from a natural source. Polypeptide molecules having substantially the same amino acid sequence as a prognostic marker but possessing minor amino acid substitutions that do not substantially affect the ability of the prognostic marker polypeptide derivatives to interact with prognostic marker-specific molecules, such as antibodies, are within the definition of prognostic marker. Derivatives include glycosylated forms, aggregative conjugates with other prognostic marker molecules, and covalent conjugates with unrelated chemical moieties. Covalent derivatives are prepared by linkage of functionalities to groups which are found in the prognostic marker amino acid chain or at the N- or C-terminal residue by means known in the art.

Antibodies specific for a prognostic marker are produced by immunizing an appropriate vertebrate host, e.g., rabbit, with purified prognostic marker or polypeptide derivatives of prognostic marker, by themselves or in conjunction with a conventional adjuvant. Usually, two or more immunizations will be involved, and blood or spleen will be harvested a few days after the last injection. For polyclonal antisera, the immunoglobulins can be precipitated, isolated and purified by a variety of standard techniques, including affinity purification using a prognostic marker attached to a solid surface, such as a gel or beads in an affinity column. For monoclonal antibodies, the splenocytes normally will be fused with an immortalized lymphocyte, e.g., a myeloid cell line, under selective conditions for hybridoma formation. For example, spleen or lymphocytes from an immunized animal can be removed and immortalized or used to prepare hybridomas by methods known to those skilled in the art. To produce a human-human hybridoma, a human lymphocyte donor is selected. Epstein-Barr virus (EBV) can be used to immortalize human lymphocytes or a human fusion partner can be used to produce human-human hybridomas. Primary in vitro immunization with peptides can also be used in the generation of human monoclonal antibodies. Antibodies secreted by the immortalized cells are screened to determine the clones that secrete antibodies of the desired specificity. Techniques for producing antibodies are well known in the literature and are exemplified by the publication Antibodies: A Laboratory Manual (1988) eds. Harlow and Lane, Cold Spring Harbor Laboratories Press, and U.S. Patent Nos. 4,381,292, 4,451,570, and 4,618,577.

The concentration of the prognostic marker in the cell sample being assayed is correlated with a standard value to determine when increased invasiveness of cells is present. The standard is usually (1) a predetermined range of prognostic marker concentrations (or other units of amount) for the same cell source in the general population as that being assayed, or (2) a previously measured prognostic marker amount or concentration from the same cell source. A lower measured concentration of prognostic marker relative to the standard value is an indication of increased invasiveness and thus poorer likely prognosis of the patient. Treatment can then be initiated or changed by the attending physician based on this information.

Diagnostic Applications using Genetic Probes

Genetic material that hybridizes with cellular genetic material associated with the prognostic markers of the invention can be used for hybridization probes in numerous assays. The analyte, usually mRNA encoding one of the prognostic markers, but also genomic DNA when mutational errors are suspected, can be a nucleotide sequence which hybridizes with a probe comprising a sequence of (usually) at least about 16 consecutive nucleotides, usually 30 to 200 nucleotides, up to substantially the full sequence of the sequences shown above (cDNA sequences). The analyte can be RNA or cDNA for full-length probes or shorter genetic materials for probes that are capable of binding to the intron regions of genomic DNA. The sample is typically a tissue or cell preparation or lysate or a bodily fluid as described herein (see also Umbas et al., 1992). A positive result is generally characterized as identifying a genetic material comprising a sequence at least about 70% homologous to a sequence of at least 12 consecutive nucleotides, preferably 20 consecutive nucleotides, more preferable 40 consecutive nucleotides, of the sequences described herein, usually at least about 80%, preferably 90%, more preferably 95%, homologous to at least about 60 consecutive nucleotides within the sequences, and may comprise a sequence substantially homologous to the full-length sequences or some part thereof.

In order to detect an analyte, where the analyte hybridizes to a probe, the probe can contain a detectable label. Probes that are particularly useful for detecting presence of the markers are based on conserved regions of prognostic

marker proteins that have been screened (either by computer analysis of sequence databases or by hybridization screening against marker-deficient cells of the type being assayed) for the absence of non-marker hybridization under the conditions being used in the assay.

One method for amplification of target nucleic acids, for later analysis by hybridization assays or as part of a detection scheme by itself, is known as the polymerase chain reaction or PCR technique. The PCR technique can be applied to detecting prognostic marker of the invention in suspected samples using oligonucleotide primers spaced apart from each other and based on the published genetic sequences of the prognostic markers. The primers are complementary to opposite strands of a double stranded DNA molecule and are typically separated by from about 50 to 450 nt or more (usually not more than 2000 nt). This method entails preparing the specific oligonucleotide primers and then repeated cycles of target DNA denaturation, primer binding, and extension with a DNA polymerase to obtain DNA fragments of the expected length based on the primer spacing. Extension products generated from one primer serve as additional target sequences for the other primer. The degree of amplification of a target sequence is controlled by the number of cycles that are performed and is theoretically calculated by the simple formula 2n where n is the number of cycles. Given that the average efficiency per cycle ranges from about 65% to 85%, 25 cycles produce from 0.3 to 4.8 million copies of the target sequence. The starting material for PCT analysis can be either DNA or RNA. Messenger RNA is a preferred target, since such mRNA is present when active expression of a prognostic marker is taking place. The PCR method is described in a number of publications, including Saiki et al., Science (1985) 230:1350-1354; Saiki et al., Nature (1986) 324:163-166; and Scharf et al., Science (1986) 233:1076-1078. Also see U.S. Patent Nos. 4,683,194; 4,683,195; and 4,683,202.

The invention includes a specific diagnostic method for determination of prognostic marker, based on selective amplification of prognostic marker-encoding DNA fragments. This method employs a pair of single-strand primers derived from non-homologous regions of opposite strands of a DNA duplex fragment selected from the published prognostic marker. These "primer fragments," which form one

aspect of the invention, are prepared from prognostic marker fragments such as described above. The method follows the process for amplifying selected nucleic acid sequences as disclosed in U.S. Patent No. 4,683,202, as discussed above.

Actions taken after evaluation of prognostic marker level

Because of the many different possible clinical goals, the action to be taken after determining a prognostic marker level indicative of invasiveness is best selected by an attending physician or veterinarian after collecting data from several samples. One can also take into consideration the potential for harm of incorrect measurements. In cases of apparent non-disseminated disease, quantitatively abnormal expression of a prognostic marker would typically suggest a more aggressive treatment than that currently being used. If metastatic disease is already established, the clinician can adjust patient expectations of prognosis according to the therapy options available, or can switch options, if no benefit is seen from the current therapy.

The method of the invention can be used to follow the prognosis of any epithelial tumor, including but not limited to colon, gastric, cervical, breast, prostate, bladder, head and neck, basal cell, thyroid, and meningioma tumors of epithelial origin.

As an alternative to comparing prognostic marker concentrations to those present in a normal population, a previously measured prognostic marker concentration of the same cell source of the same patient can be used as a standard for comparison. In this case, what is being determined is usually the rate of increase or decrease in prognostic marker concentration as an indication of the progression of the disease or the success of the treatment. A positive assay (i.e., indication of decreased invasiveness) is considered to be present when the measured concentration exceeds a previously measured prognostic marker concentration made in the same cell source of the same patient. For example, a patient who previously showed only 50% positive marker cells in a tissue sample might show 75% positive after a particular treatment (e.g., chemotherapy with a given drug, where previous chemotherapy with different drugs did not show improvement in presence of these prognostic markers). Again the selection of a particular change as indicative of a

change in treatment is best selected by the attending physician for the particular reason desired. Increases in prognostic marker concentration that meet the standards of this paragraph and additionally reach the essential homogeneous positive patterns of markers found in normal populations of patients are particularly indicative of appropriate treatment.

It will be recognized by those skilled in clinical analysis that assays for a given analyte, including this assay for a prognostic marker, are not expected to be obtained or to be interpreted by an attending physician in the absence of additional information. Additionally, the results of any assay are best considered to be indicative of the probability of the presence of a clinical condition rather than as absolute proof. The same situation exists for the present invention. Nevertheless, an indication of increased cell invasiveness is clinically useful information and can be used by a skilled medical practitioner in combination with other information to care for patients in a more informed manner than would be possible if the information were not available.

By monitoring concentration or amounts of prognostic markers of the invention, in addition to following the progress of a disease and modifying treatment as necessary, one can also monitor the success (or failure) of various treatments. It has been demonstrated in the laboratory of the present inventors that a redifferentiation drug that induces differentiation of tumor cells also increases expression of E-cadherin. Thus, one can monitor progress of treatment in the same manner that one can monitor progress of the disease. Such treatment include but are not limited to chemotherapy and surgery. The necessity of treatments considered more or less radical by the medical profession can be selected by a physician using the monitoring techniques of the invention in combination with other criteria of clinical status.

Monitoring Prognosis Markers as a Screen for Dedifferentiation Drugs

Compounds can be evaluated for potential redifferentiation activity for epithelial cells by adding a test compound to a cell composition deficient in expression of E-cadherin or α-catenin, monitoring the cell composition for increased expression of the appropriate marker, and selecting the test compound for use or

additional testing as a redifferentiation compound if the cell composition shows increased expression of the marker. Any of the techniques described above can be applied to the monitoring process. The epithelial cell line deficient in marker expression can be selected on the basis of prior knowledge of marker expression or can readily be selected from epithelial cell tumor lines (e.g., obtainable from the American Type Culture Collection, Rockville, Maryland, USA, as well as numerous other cell line repositories) using the same assays for the prognostic markers described herein. One cell line that has been demonstrated to respond in this manner is Dunning R3327 AT6, but the invention is not limited to this example.

The invention now being generally described, the same will be better understood by reference to the following detailed examples, which are provided for illustration only and are not to be considered limiting of the invention unless otherwise stated.

EXAMPLES

Example 1: Clinical Evaluation of Effects of Decrease in E-Cadherin Expression

Part 1: Patients

Eighty-nine prostate cancer patients, treated between January 1987 and December 1991, were included in a study. The mean age at the time of diagnosis was 70 years (range: 42 to 87 years). Clinical tumor staging was done by digital rectal examination and transrectal ultrasound. Pathological staging was determined according to the classification of the International Union Against Cancer (see Harmer, M.H. TNM Classification of malignant tumours, 3rd ed. Geneva: International Union Against Cancer, 1978). Bone scan was performed in order to detect metastatic lesions and further evaluation by radiographs was done in case of a positive bone scan. The treatment options were radical prostatectomy for early stage and hormonal therapy, either medical or surgical, for advanced disease. Only in 4 cases external beam radiotherapy with linear acceleration was given in combination with hormonal treatment. The patient characteristics are summarized in Table 1.

Table 1: Patients characteristics (at diagnosis, n= 89)

	No(%)
Gleason score:	
4	11(12)
5 - 7	39(44)
8 - 10	39(44)
Clinical stage:	
T1	17(19)
T2	29(32)
T3	30(34)
T4	13(15)
Metastasis:	
No metastasis	59(66)
With metastasis	29(33)
Unknown[a]	1(1)
Initial treatment:	
Primary[b] TUR-P[c]	29(33)
TUR-P after recurrent disease	18(20)
Radical Prostatectomy[d]	42(47)

[a]: Bone scan was not done in one patient with T4 tumor.
[b]: Followed by hormonal treatment and/or radiotherapy (in 4 cases).
[c]: TUR-P, Transurethral resection of the prostate.
[d]: Including 3 patients with pre-operative adjuvant hormonal therapy.

Part 2: Surgical specimens

Transurethral resection or radical prostatectomy specimens were snap frozen. Sections of 4-6 μm thickness were cut on a cryostat, air-dried, and stored at -20°C until use. One section from each patient was stained with hematoxylin and eosin to assess the histopathological grade according to Gleason (Gleason, D.F. Histologic grading and clinical staging of prostatic carcinoma. *In:* M. Tannenbaum (ed.), Urologic Pathology: The Prostate, pp. 171-197. Philadelphia: Lea and Febiger, 1977). In the group of patients described in Part 1 of Example 1, tumor grade ranged from Gleason score 4 to 10.

Part 3: Immunohistochemistry

Indirect immunoperoxidase staining was performed as described in Umbas, et al. 1992, using either anti E-cadherin (Eurodiagnostica BV, Apeldoorn, The Netherlands) or HECD-1 (Takara, Berkeley, U.S.A.) monoclonal antibodies. E-cadherin staining is localized on the membrane, particularly at areas of cell-cell contact. To assess the staining we used the following criteria: uniformly positive, uniformly negative or heterogeneous (mixed populations of positive and negative stained cells) as described by Schipper et al. Besides positive or negative staining, some tumors showed a cytoplasmic staining, which was also considered to be abnormal and included in the criteria for heterogeneous staining. Uniformly positive staining patterns were regarded as normal while uniformly negative and heterogeneous stainings were considered as aberrant expression.

Part 4: Statistical analysis

The correlation of E-cadherin expression with tumor grade, clinical stage, pathological stage and metastases was evaluated by the chi-square test readjusted where necessary with the Yates correction. The value of significance was taken as $p<0.055$. Actuarial survival rate of patients with normal and decreased E-cadherin expression were evaluated according to Kaplan-Meier (Kaplan, E.L., and Meier, P. Nonparametric estimation from incomplete observations. J. Amer. Stat. Assoc., *53:* 457-481, 1958), and the differences were tested with log-rank test. The statistical

software used was Statistical Package for the Social Sciences (SPSS/PC+ 4.0, Chicago, USA).

Part 5: Analysis results

Eighty-nine snap frozen prostate cancer specimens were stained with the anti-E-cadherin monoclonal antibodies anti-E-cadherin or HECD-1 and scored as described in Part 3 of Example 1. The scoring system is based on the biological functional relation between loss of E-cadherin expression and invasiveness, i.e., the presence of a negative subpopulation is considered to have important biological significance. In Figure 1a, an example of normal expression can be seen with uniformly positive staining on the membrane at cell-cell contacts. Abnormal patterns comprise partially positive and partially negative stainings on the membrane or a cytoplasmic staining (Fig. 1b). Occasionally, no E-cadherin expression at all was found in the entire tumor area evaluated (Fig. 1c). Both of the latter staining patterns were regarded as aberrant expression of this molecule.

We also evaluated whether aberrant E-cadherin expression correlated with stage and the presence of metastasis. Indeed, there was a correlation between degree of local extension of the tumor and aberrant expression of E-cadherin (Table 2). All well differentiated tumors (n=11) showed normal expression, while 28% and 79% of the moderately and poorly differentiated tumors respectively showed aberrant E-cadherin expression. The correlation between aberrant E-cadherin expression and local extension of the tumor (clinical tumor stage) was statistically significant ($p < 0.005$). Only 33% of the T1-2 tumors (organ confined) showed aberrant E-cadherin expression compared to 63% of T3-4 tumors (locally invasive) (Table 2). Moreover, the presence of metastases was significantly correlated with aberrant E-cadherin expression (Table 2, $p < 0.001$). In tumor tissue from patients without metastatic disease E-cadherin expression was normal in 68% and aberrant in 32%. On the other hand, E-cadherin expression was normal in 24% and aberrant in 76% of the patients that presented with metastases (Table 2).

Table 2: Relationship of E-cadherin expression to tumor grade, clinical stage and metastases.

	E-cadherin expression				
	Normal No.	(%)	Decreased No.	(%)	Significance of difference
Gleason score:					
4	11	(100)	0	(0)	p <0.001
5 - 7	28	(72)	11	(28)	x2= 31.8
8 - 10	8	(21)	31	(79)	
Clinical stage:					
T1-2	31	(67)	15	(33)	p<0.005
T3-4	16	(37)	27	(63)	x2= 8.1
Metastases:					
-	40	(68)	19	(32)	p<0.001
+	7	(24)	22	(76)	x2= 14.9

Part 6: Survival studies

We also investigated the relation between E-cadherin expression and survival. The average follow up time for patients who were still alive at the time of evaluation was 36 months (range: 12 to 71 months). The 3 year over-all survival rate of patients with normal E-cadherin expression was significantly higher than for patients with aberrant expression [(log rank test: chi-square=20.4, $p<0.001$ (Fig. 2)]. The group of patients described above is heterogenous; accordingly we stratified according to treatment, *i.e.* radical prostatectomy *versus* palliative TUR-P.

The characteristics of the patients treated by radical prostatectomy are summarized in Table 3. Eleven of 42 patients (26%) had evidence of disease progression (clinical or biochemical progression, *i.e.*, PSA$\geq$0.5ng/ml) after 8 to 42 months. All of them but one (91%) showed aberrant E-cadherin expression in their tumor specimens (Table 5). Further analysis showed a significant correlation (log rank test: chi-square=9.4, $p<0.005$) between aberrant E-cadherin staining and progression as presented in Figure 3.

Table 3: Characteristics of patients who underwent radical prostatectomy (n= 42).

No.	Preop.		Final pathology			Recurrence		Survival
	Clin. Stage	Gleason Score	Caps. penet.	Surg. margin	Lymph nodes	months post.op.	PSA (ng/ml)	months
				Normal E-cadherin expression				
1.	2	4	-	-	-	-	<0.5	41A
2.	1	4	-	-	-	-	<0.5	57A
3.	2	4	-	-	-	-	<0.5	21A
4.	1	4	-	-	-	-	<0.5	27A
5.	2	4	-	-	-	-	<0.5	36A
6.	2	4	-	-	-	-	<0.5	27A
7.	2	4	-	-	-	-	<0.5	57A
8.	2	4	-	-	-	-	<0.5	28A
9.	2	5	+	-	-	-	<0.5	41A
10.	2	5	-	-	-	-	<0.5	20A

Table 3 (cont): Characteristics of patients who underwent radical prostatectomy.

No.	Preop.		Final pathology			Recurrence		Survival
	Clin. Stage	Gleason Score	Caps. penet.	Surg. margin	Lymph nodes	months post.op.	PSA (ng/ml)	months
11.	2	5	-	-	-	-	<0.5	24A
12.	2	6	-	-	-	-	<0.5	59A
13.	1	6	-	-	-	-	<0.5	40A
14.	1	6	+	-	-	-	<0.5	27A
15.	2	6	-	-	-	-	<0.5	41A
16.	2	6	-	-	-	-	<0.5	57A
25.	1	6	-	-	-	-	<0.5	15A
27.	1	6	-	-	-	-	<0.5	23A
20.	2	7	-	-	-	--	<0.5	47A
22.	1	7	-	-	-	-	<0.5	23A
23.	1	7	+[a]	-	-	-	<0.5	15A
26.	1	7	-	-	-	-	<0.5	14A

Table 3 (cont): Characteristics of patients who underwent radical prostatectomy.

No.	Preop.		Final pathology			Recurrence		Survival
	Clin. Stage	Gleason Score	Caps. penet.	Surg. margin	Lymph nodes	months post.op.	PSA (ng/ml)	months
17.	2	8	-	-	-	-	<0.5	45A
18.	2	8	-	-	-	-	<0.5	37A
19.	2	8	+	+	-	-	<0.5	65A
24.	1	8	-	-	+	36	0.5	36B
21.	2	9	+[a]	-	+	-	<0.5	35A

Table 3 (cont): Characteristics of patients who underwent radical prostatectomy.

No.	Preop.		Final pathology			Recurrence		Survival
	Clin. Stage	Gleason Score	Caps. penet.	Surg. margin	Lymph nodes	months post.op.	PSA (ng/ml)	months
				Decreased E-cadherin expression				
1.	1	5	-	-	-	-	<0.5	47A
2.	1	5	-	-	-	-	<0.5	35A
3.	1	6	+	-	-	42	5.3	65B
4.	1	6	-	-	-	-	<0.5	35A
5.	1	7	+[a]	-	+	-	<0.5	47A
6.	1	7	-	-	-	-	<0.5	35A
7.	2	7	+[a]	-	+	31	4.9	33B
8.	3[b]	8	-	-	-	23	1.5	31B
9.	2	8	-	-	-	31	1.8	36B
10.	2	9	+	-	-	28	1.1	37B

Table 3 (cont): Characteristics of patients who underwent radical prostatectomy.

No.	Preop.		Final pathology		Recurrence			Survival
	Clin. Stage	Gleason Score	Caps. penet.	Surg. margin	Lymph nodes	months post.op.	PSA (ng/ml)	months
11.	2[b]	9	+	-	-	10	1.6	24B
12.	2	9	-	-	-	40	1.0	40B
13.	2	9	+[a]	-	-	17	71.1	17B
14.	2[b]	10	+	+	-	8	<0.5	16D
15.	3	10	+	-	+	37	3.5	46B

[a]: With seminal vesicle involvement.

[b]: After pre-operative adjuvant hormonal therapy.

A= Alive without disease.

B= Alive with disease / recurrence.

D= Death with disease.

L= Lost of follow up.,

Among the patients who have capsular penetration ($pT \geq 3$), 8 had aberrant E--cadherin expression and 7 out of them progressed while 5 had normal E-cadherin expression and only one of them progressed (Table 3).

Forty-seven patients with advanced disease were treated by transurethral resection of the prostate (TUR-P). We can divide this group into primary TUR-P followed by hormonal treatment and/or occasionally radiotherapy and TUR-P after hormone escaped disease (Table 1).

In the primary TUR-P group, the analysis of survival within 3 year after tumor resection shows that aberrant staining is significantly correlated with poor prognosis [log rank test: chi-square=15.1, $p<0.001$ (Fig. 4a)].

The mean survival of patients with hormone escaped disease who underwent a TUR-P was 25 months for patients with a normal E-cadherin expression and 17 months for patients with a decreased expression. However, the 3 year survival analysis of this group shows no significant correlation between decreased immunoreactivity and survival [log rank test: chi-square=1.2, $p<0.3$ (Fig. 4b)].

<u>Example 2: Clinical Evaluation of Effects of Decrease in E-Catenin Expression</u>

Part 1: Patients

Twenty early stage prostate cancer patients and 32 advanced stage patients were included in this experiment. Treatment options were radical prostatectomy for early stage and hormonal treatment for advanced disease. Follow-up was done by measuring prostate specific antigen level, and bone scan was performed to detect metastatic lesions.

Part 2: Surgical specimens.

All tissues were obtained at the time of surgery (either radical prostatectomy or transurethral resection) and snap frozen. Four to six mm serial sections from the frozen tissues were cut on cryostat and air dried, then stored at -20°C until use. One section from each patient was stained with hematoxylin and eosin to determine the histopathological grading using the Gleason score system.

Part 3: Antibodies.

L-CAM (uvomorulin) monoclonal antibody against E-cadherin (Eurodiagnostica BV, Apeldoorn, The Netherlands) and α-18, a rat monoclonal antibody against α-catenin (Shimoyama 1992), were used in this study.

Part 4: Immunohistochemistry.

Immunohistochemistry was performed by using indirect method as described previously (Umbas 1992) except that we used biotinylated (Amersham) anti-mouse Ig for E-cadherin and anti-rat Ig for α-catenin staining as the second antibody and incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA) before incubation with diaminobenzidine 0.6 mg/ml in 0.65% Imidazol/Phosphate-buffered saline. E-cadherin and α-catenin staining patterns were scored as described in Example 1; uniformly positive staining was regarded as normal, while heterogeneous, uniformly negative, and cytoplasmic stainings were scored as aberrant staining.

Part 4: Analysis results

As reported in Example 1, there was significant correlation between E-cadherin expression and tumor grade, tumor stage, survival of the advanced stage patients, and progression free interval in patients with low stage disease treated by radical prostatectomy.

In this Example we found significant correlation between α-catenin and E-cadherin expression (Table 4). There were 3 staining combinations: first, normal expression of both E-cadherin and α-catenin; second, normal E-cadherin expression and aberrant α-catenin staining; and third, both aberrant expression of E-cadherin and α-catenin. Taking together, patients who showed both normal immunoreactivity of these molecules had significantly better prognosis than patients having aberrant α-catenin expression even if the E-cadherin expression was normal (Table 5).

In the radically treated group, 4 patients showed aberrant E-cadherin and α-catenin expression, and all of these patients progressed within 8 to 37 months. The other 16 patients of this group showed normal expression of both E-cadherin and α-catenin, and no progression could be detected in a mean follow up of 40 months (range; 20-65 months).

Thirteen patients with advanced disease had normal E-cadherin expression; nine of these patients also had normal α-catenin expression. Two (22%) of them died of the disease while 2 (50%) of the 4 patients in this group who had aberrant α-catenin expression died. Of the other 19 patients who had both aberrant expression of E-cadherin and α-catenin, only 3 (16%) were alive without progression, while the other 16 (84%) either developed progression (1 patient) or died of the disease.

Table 4: Correlation between E-cadherin and α-catenin expression.

	α-catenin	
	Normal	Aberrant
E-cadherin		
Normal	25	4
Aberrant	0	23

$x^2 = 38.2$

Table 5: Correlation between E-cadherin and α-catenin expression and progression (n=52).

	Progression +	Progression -
Normal E-cadherin and α-catenin	23	2
Normal/aberrant E-cadherin aberrant α-catenin	5	22

$x^2 = 28.2$

Table 6: Relationship of α-catenin expression to tumor grade.

	α-catenin expression			
	Normal		Aberrant	
	No.	(%)	No.	(%)
Gleason score				
4	8	(100)	0	(0)
5 - 7	15	(71)	6	(29)
8 - 10	2	(9)	21	(91)

Table 7: Correlation between α-catenin expression and survival of advanced cases with normal E-cadherin expression (n=13).

	Survival	
	Alive	Death
Normal α-catenin	7	2
Aberrant α-catenin	2	2

Table 8: Correlation between E-cadherin and α-catenin in advanced cases (n=32).

	Patients outcome		
	Progression (-)		Progression (+)
Normal E-cadherin and α-catenin (22%)	7	(78%)	2
Normal E-cadherin and aberrant α-catenin (50%)	2	(50%)	2
Aberrant E-cadherin and α-catenin (84%)	3	(16%)	16

<u>Example 3: Evaluation of redifferentiation drugs using E-cadherin expression as a diagnostic</u>

The results of Examples 1 and 2 suggested to us the potential for using these prognosis markers in model systems used to evaluate candidate drugs for their ability to induce reexpression of the marker and thus dedifferentiation of tumors. Treatment of the Dunning R3327 AT6 epithelial tumor subline with liarozole (Jansen Research Foundation, Beerse, Belgium) resulted in the induction of squamous differentiation in this anaplastic tumour and a concomitant reexpression of E-cadherin mRNA. This was associated with reexpression of the E-cadherin gene; hence an assay for the E-cadherin promotor, an easy and quick procedure, is demonstrated to be useful as a screening tool for dedifferentiation drugs.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WE CLAIM:

1. A method for determining invasiveness potential of an epithelial tumor, which comprises:

determining a prognostic amount of a prognostic marker selected from the group consisting of E-cadherin and α-catenin in a cell sample obtained from a cell source containing cells of said tumor; and

comparing said prognostic amount to a normal amount of said prognostic marker in said cell source, wherein when said prognostic amount is less than said normal amount, said sample is indicative of invasiveness potential of said cells of said prostate tumor.

2. The method of Claim 1, wherein said prognostic marker is E-cadherin.

3. The method of Claim 1, wherein said prognostic marker is α-catenin.

4. The method of Claim 1, wherein said prognostic marker is both E-cadherin and α-catenin.

5. The method of Claim 1, wherein said determining comprises a specific binding assay for said prognostic marker.

6. The method of Claim 1, wherein said determining comprises a hybridization assay for a genetic precursor of said marker.

7. The method of Claim 1, wherein said comparing is measured by immunohistostaining of said sample.

8. The method of Claim 1, wherein said epithelial tumor is a prostate tumor.

9. A method of screening a compound for potential redifferentiation activity for epithelial cells, which comprises:

adding said compound to a cell composition deficient in expression of a cell marker selected from E-cadherin and α-catenin,

monitoring said cell composition for increased expression of said marker, and

selecting said compound for use or additional testing as a redifferentiation compound if said cell composition shows increased expression of said marker.

Fig.1a.

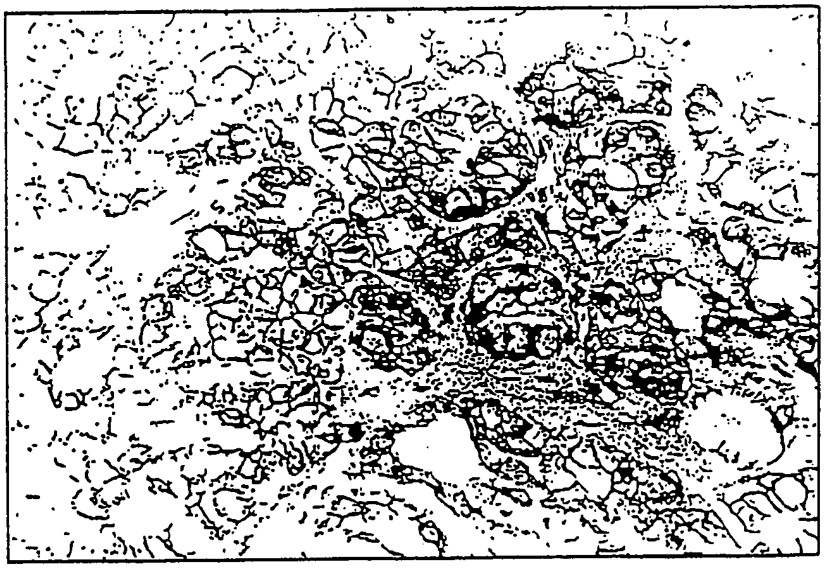


Fig.1b.

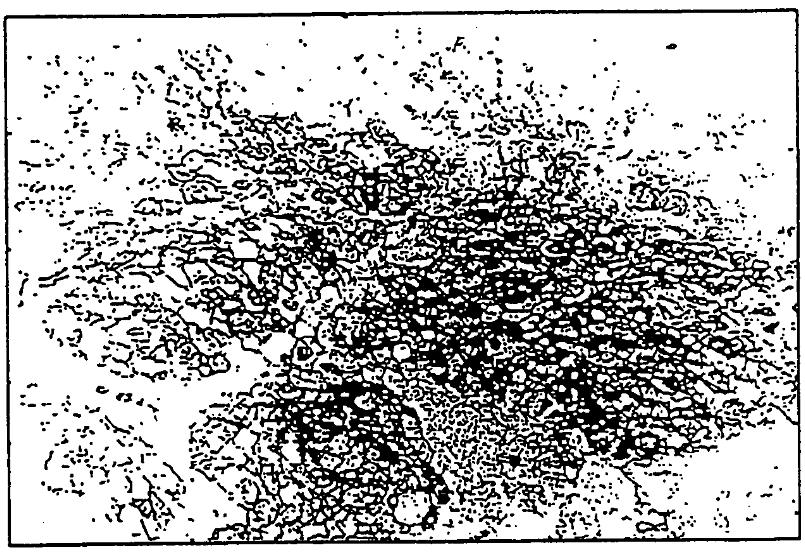


Fig.1c.

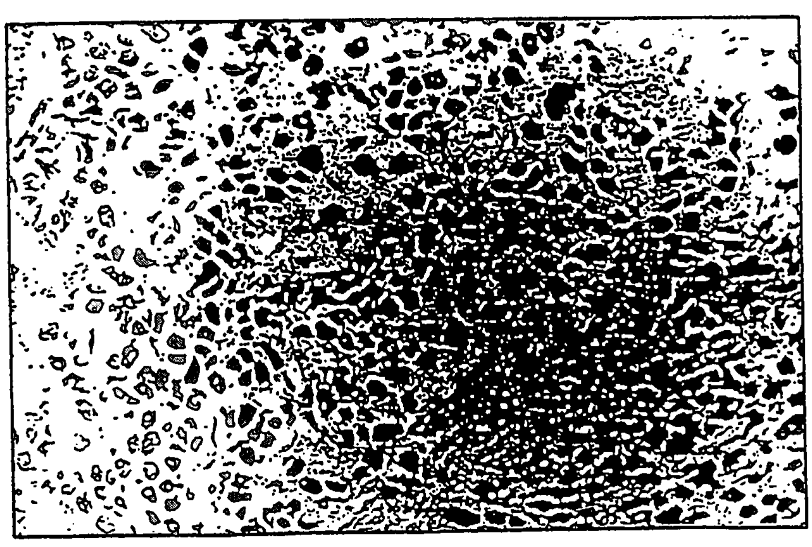
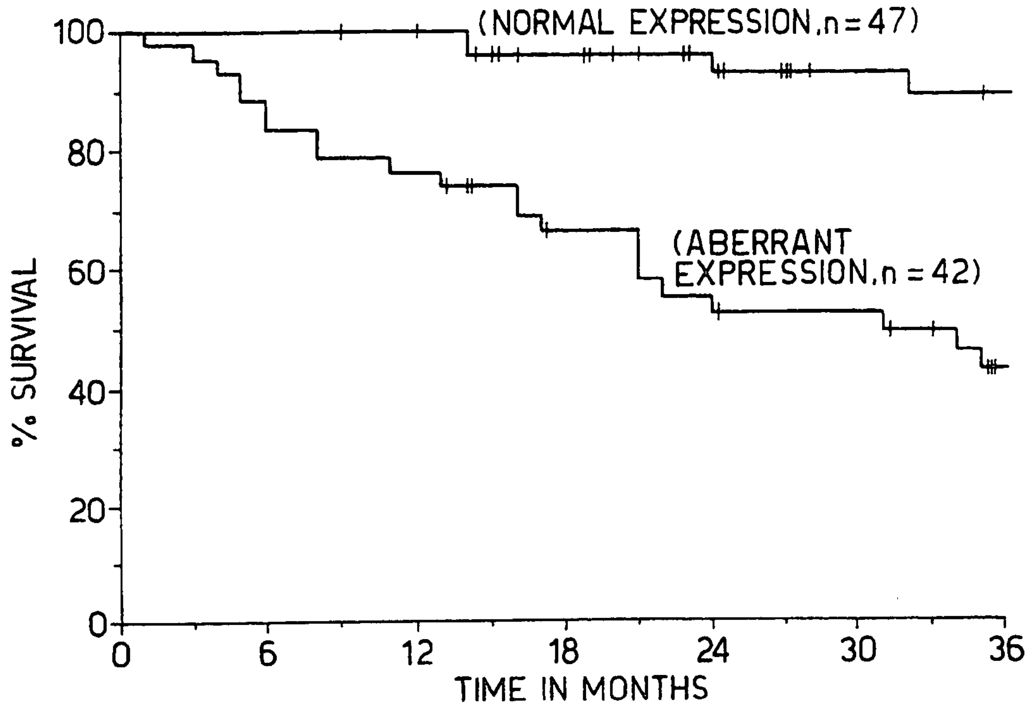


Fig.2.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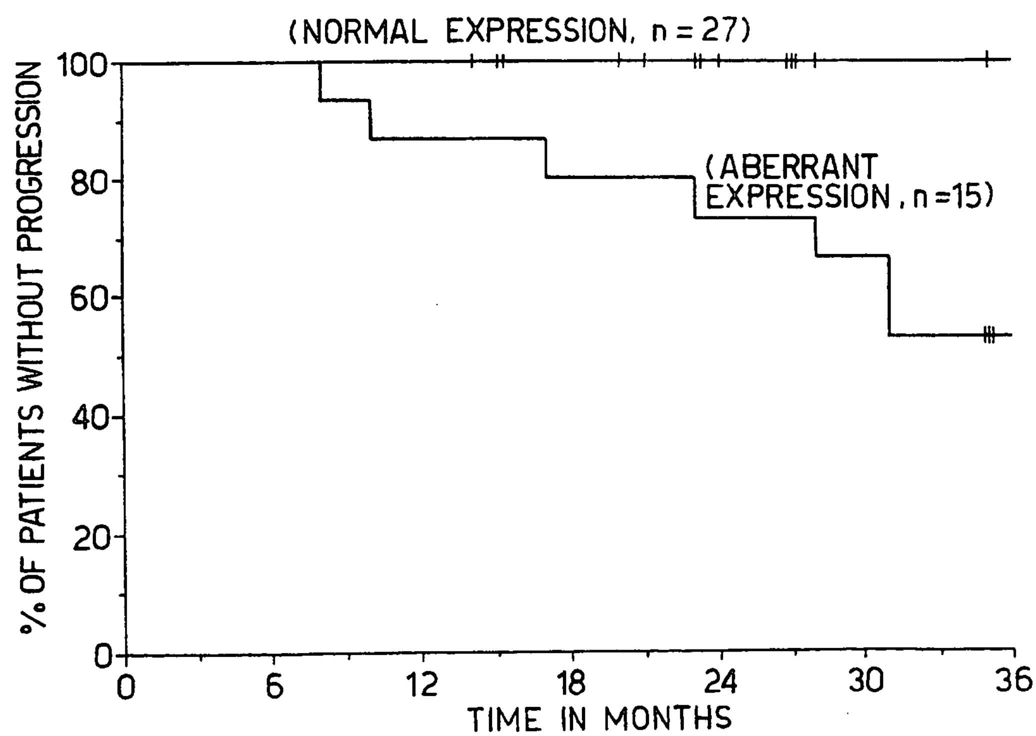
TIME IN MONTHS

Fig.3.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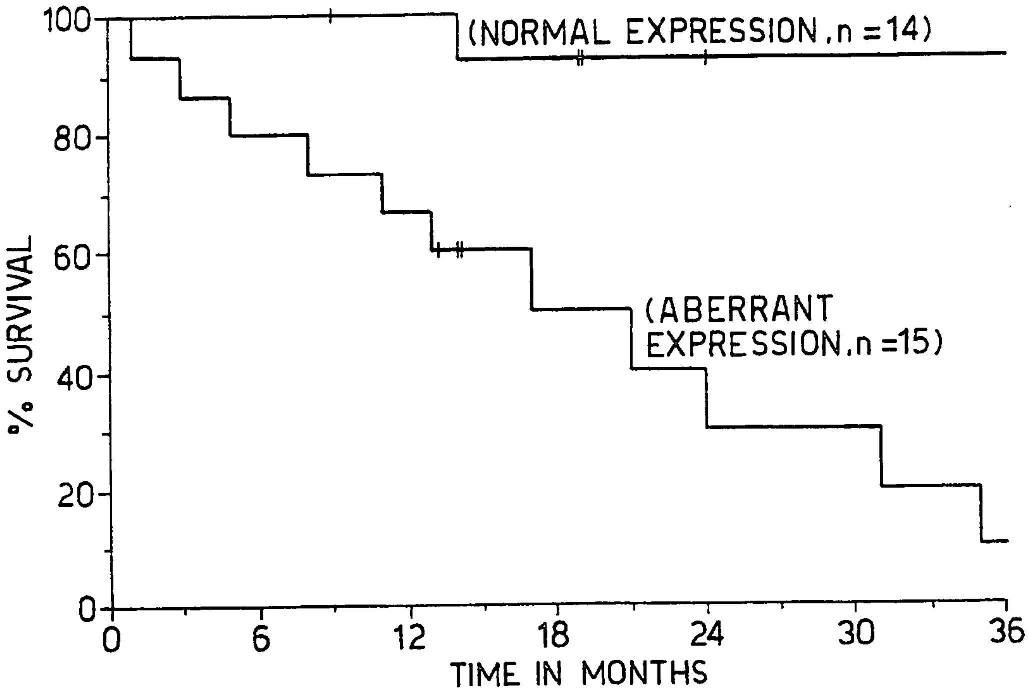
TIME IN MONTHS

Fig.4a.



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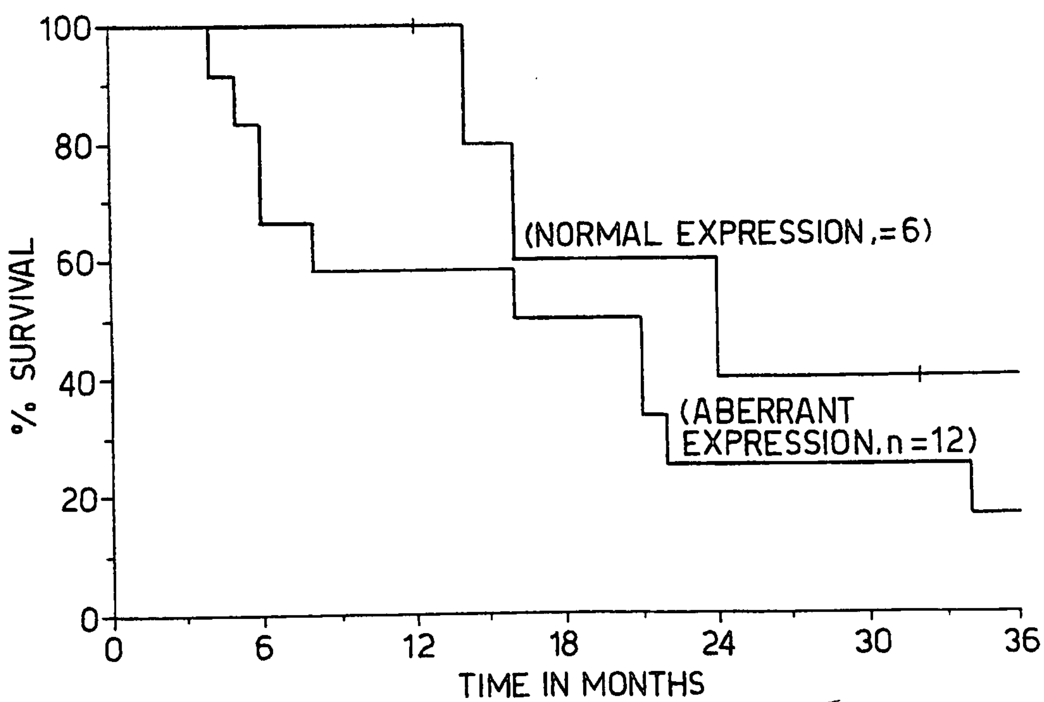
TIME IN MONTHS

Fig.4b.



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TIME IN MONTHS

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 95/00577

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/574 C12Q1/68 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 17608 (W. BIRCHMEIER) 15 October 1992 see the whole document ---	1,2,4-7
X	DATABASE WPI Section Ch, Week 9410 Derwent Publications Ltd., London, GB; Class B04, AN 94-079303 & JP,A,06 030 785 (EISAI CO. LTD.) , 8 February 1994 see abstract ---	1,3-5,7
X	WO,A,94 11401 (26-05-94) 26 May 1994 see the whole document -----	1,2,4-9

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
27 October 1995	15. 11. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Griffith, G

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 95/00577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9217608	15-10-92	DE-A- 4110405	01-10-92
		EP-A- 0582582	16-02-94
		JP-T- 7500723	26-01-95
WO-A-9411401	26-05-94	AU-B- 5669494	08-06-94